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Complete nucleotide sequence of the haemagglutinin gene from a human influenza virus of the Hong Kong subtype

G.W.Both and M.J.Sleigh

CSIRO, Molecular and Cellular Biology Unit, P.O. Box 184, North Ryde, N.S.W. 2113, Australia

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ABSTRACT

The complete nucleotide sequence has been determined for a cloned double-stranded DNA copy of the haemagglutinin gene from the human influenza strain A/NT/60/68/29C, a laboratory-isolated variant of A/NT/60/68, an early strain of the Hong Kong subtype. The gene is 1765 nucleotides long and contains information sufficient to code for a protein of 566 amino acids, which includes a hydrophobic leader peptide (16 residues), HA1 (328), HA2 (221) and an arginine residue which joins the HA subunits. Comparison of the predicted amino acid sequence for 29C haemagglutinin with protein sequence data available for HA from other influenza strains shows that no potential coding information is lost by processing of the mRNA.

A comparison of the amino acid sequences predicted from the gene sequences for 29C and fowl plague virus haemagglutinins, (1) indicates the extent to which changes can occur in the primary sequence of different regions of the protein, while maintaining essential structure and function.

INTRODUCTION

The genome of influenza A virus is segmented and consists of eight single stranded RNA species of negative polarity. The fourth largest segment codes for the viral haemagglutinin (HA) and the sixth for neuraminidase (2-7). The virus is notable for the frequency with which alterations in these two surface proteins are observed, changes in their structure resulting in changes in viral antigenic character. Antigenic shift occurs when there is a radical change in the antigenicity of the surface proteins leading to the appearance of a new viral subtype, while antigenic drift results from smaller, progressive changes in antigenicity within a subtype (8).

In an attempt to relate changes in viral antigenicity to changes in the primary structure of the major antigenic protein, haemagglutinin, peptide maps and amino acid sequences of this protein prepared from different viral

strains have been compared (9,10). However, the development of techniques for cloning double-stranded (ds) DNA copies of RNA genes and for rapid nucleotide sequenc ing has made it easier to study antigenic variation at the level of the nucleic acid. As a prelude to comparative sequence analysis of influenza HA genes, we synthesized a dsDNA copy of the HA gene and cloned it by insertion into the plasmid pBR322, amplified in E. coli RRI (7,11). Here we report the complete sequence of the HA gene from influenza strain A/NT/60/68/29C, a laboratory-derived mutant produced from A/NT/60/68, an early field isolate in the Hong Kong subtype (12,13).

MATERIALS AND METHODS

Growth and Purification of Virus. The virus strain A/NT/60/68/29C, supplied by Dr. C. Hannoun was grown and purified by Drs. V. Bender and B. Moss, as previously described (11).

Synthesis, cloning and characterisation of a dsDNA copy of the HA gene. Procedures for the extraction of viral RNA, the synthesis of a dsDNA copy of the HA gene, its insertion into pBR322 and amplification in E. coli RRI have been described (7,11). (All recombinant DNA experiments were carried out under CII-EKI conditions as prescribed by the Recombinant DNA Committee of the Australian Academy of Science). The sequence inserted into pBR322 in clone C89 was previously identified as an authentic copy of the HA gene by comparing the nucleotide sequence of a small section (7) with the amino acid sequence determined for the corresponding region of the HA protein of the influenza strain A/Mem/102/72 (14).

Preparation of labelled restriction fragments. Plasmid DNA prepared from clone C89 (7,11) was digested for two hours with restriction enzymes in 10µl of buffer containing Tris-HCl, pH7.4 (6mM), NaCl (20mM), $MgCl_2$ (6mM), 2-mercaptoethanol (6mM) and 0.1 mg/ml bovine serum albumin. After digestion, the mixture was adjusted to give a concentration of Tris-HCl, pH 8.0 (55mM), KCl (40mM) and three unlabelled deoxynucleoside triphosphates (each 40µM). This solution was incubated for 15 min. at 37° with 10-20µCi of the fourth deoxynucleoside triphosphate, α^{32}P-labelled, and 1µl (approx. 8 units) of AMV reverse transcriptase (kindly supplied by Dr. J.W. Beard, Life Sciences, Inc., St. Petersberg, Fla.). Restriction enzymes used for digestion were chosen such that only one end of the required DNA fragment could be labelled under the above conditions. Alternatively, after labelling, the digestion mixtures were heated to inactivate reverse transcriptase (70°, 15 min) and an unlabelled excess (1mM) of the radioactive deoxynucleoside triphosphate was

added. A second restriction enzyme digestion was then carried out. Labelled fragments were separated by electrophoresis on a 4% polyacrylamide gel (11) together with labelled restriction fragments of known size as markers. Appropriate fragments were extracted from the gel and sequenced by the method of Maxam and Gilbert (15).

Determination of gene sequence directly from viral RNA. The sequence at the 5' end of the HA gene, not represented in C89, was determined by the method of Sanger et al, (16) using a denatured restriction fragment from C89 to prime DNA synthesis, with viral genome RNA as template (17).

Compilation and analysis of sequence data. Nucleotide sequence data were stored and analysed in a Digital PDP 11/10 computer, using programmes devised by Staden (18,19), kindly adapted for our system by Caroline Bucholtz and Dr. Alex Reisner. The HA proteins from fowl plague virus (FPV) and the Hong Kong subtype were compared using the hydrophobicity values for amino acids (20,21) as described by Bigelow (22) and computer programmes devised by Dr. Alex Reisner.

RESULTS

Characterisation of the cloned ds DNA copy of the HA gene from influenza strain A/NT/60/68/29C (7) included the derivation of a restriction map. This information was used to prepare suitable restriction fragments for nucleotide sequence analysis, resulting in the sequencing strategy shown in Fig. 1. Since data were available on the amino acid sequence of areas of the HA protein from another Hong Kong-type virus, A/Mem/102/72 (14), approximately 60% of the

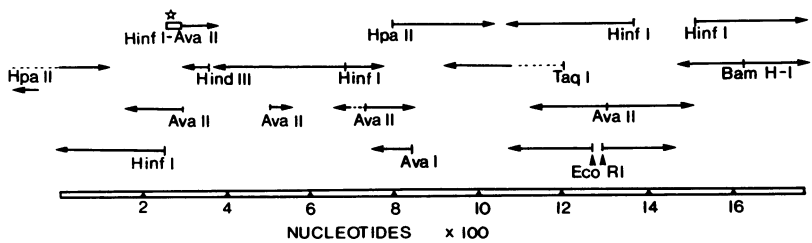


Figure 1. Strategy for sequencing a cloned dsDNA copy of the HA gene from strain 29C. The arrow shows the amount and the direction of the composite sequence information obtained from multiple experiments. (☆) The sequence of bases 300-370 was obtained using the Sanger chain termination method (16) copying the HA gene RNA into cDNA using the Hinf I - Ava II fragment as a primer for reverse transcriptase (17).

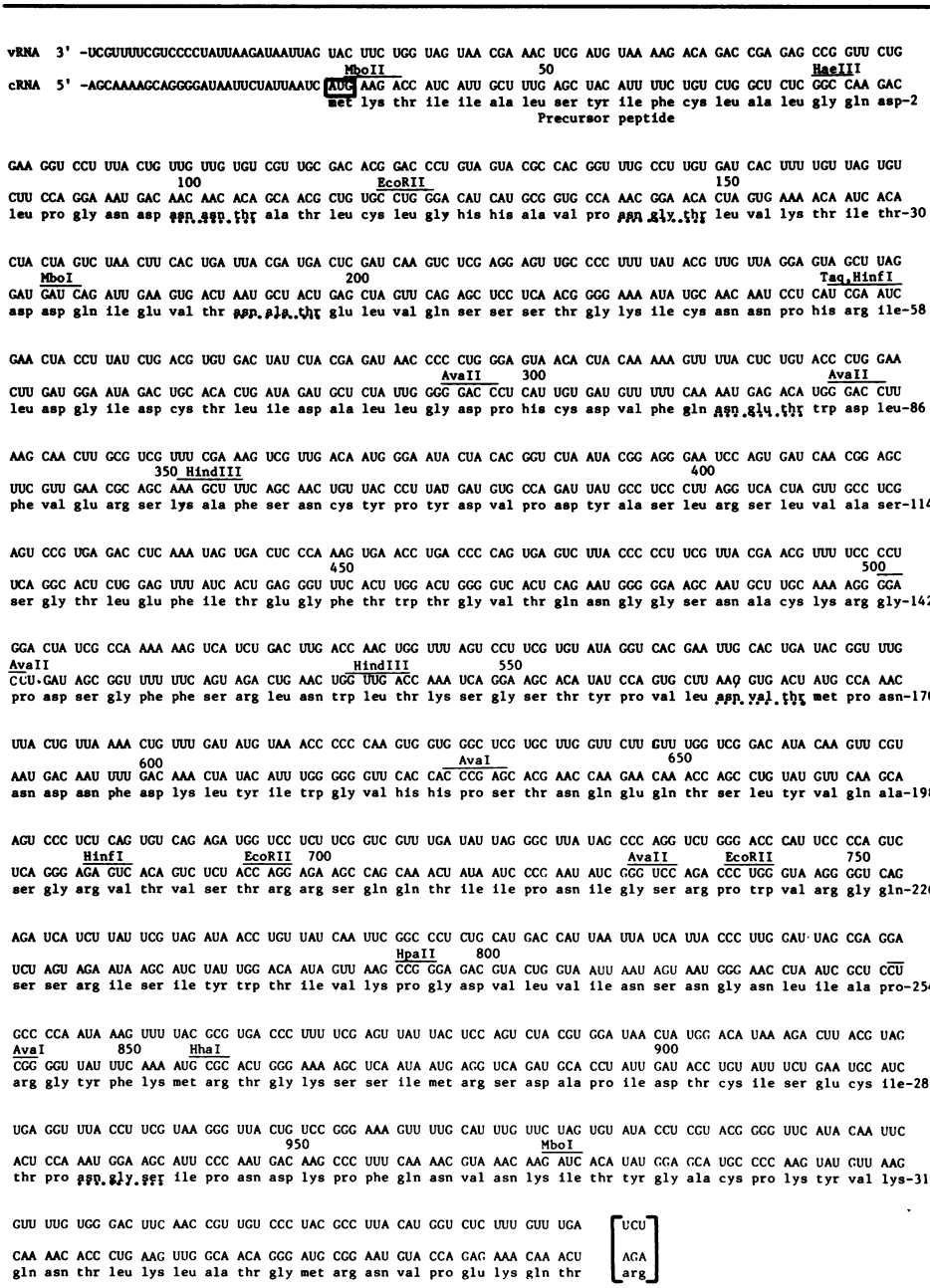


Figure 2a. For legend see over page.

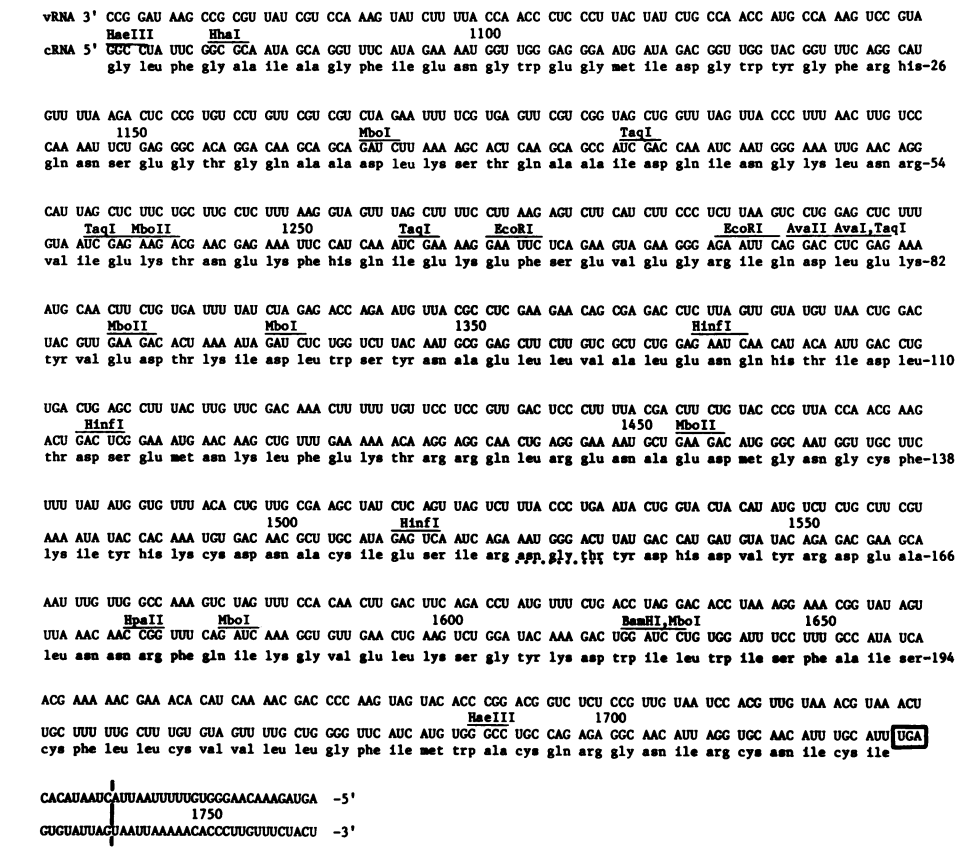


Fig 2b

Figure 2. Nucleotide sequence of the HA gene from Hong Kong influenza strain 29C and the amino acid sequence predicted from it. The RNA sequence ((-) strand) is shown from 3' → 5' below it, the complementary (+) strand representing the mRNA sequence. Initiation and termination codons are boxed and the arginine residue which connects HA1 (Fig. 2a) and HA2 (Fig. 2b) is bracketed. Possible glycosylation sites in the protein are underlined with dots. The end of the clone is indicated by the vertical line to the right of the termination codon. Restriction sites in the plasmid DNA are indicated in the equivalent position on the mRNA sequence.

gene copy was sequenced on one DNA strand only. Adjoining sections of sequence overlapped by a minimum of 15 nucleotides, except in the region of the Hind III site (base 353), where the sequence was confirmed from the viral RNA itself, using the chain termination sequencing method (16). A denatured 51-base DNA fragment, obtained by digestion of C89 DNA with Hinf I and Ava II, was used as

a primer for DNA synthesis (17). A similar technique was used in an attempt to obtain the 5' terminal gene sequence, which was not represented in the cloned gene (7).

Figure 2 shows the nucleotide sequence determined for the cloned dsDNA copy of the HA gene from strain 29C and the amino acid sequence predicted for its protein. The cloned gene copy contains 1739 nucleotides, commencing from the 3' terminal base of the gene, with the first 12 bases identical to the common sequence found at the 3' termini of other influenza genome segments (23,24). The cloned sequence extends nine bases beyond a termination codon in the same phase as the only reading frame that is continuous for the length of the gene. Part of the sequence shown for the 5' terminal region of the gene beyond the end of the clone must be regarded as tentative. The sequence shown is identical to that obtained from a cloned copy of this section of the HA gene from the 29C parent strain, A/NT/60/68 (25). Attempts to determine the sequence in this region directly from the 29C viral RNA gave clear results between bases 1734-1744 and 1752-1763, the latter segment lying within a sequence common to the 5' termini of all influenza genes so far examined (23,24). This leaves in doubt a section of 7 nucleotides, whose sequence appeared to be the same as that in A/NT/60/68, but for which unequivocal data could not be obtained (data not shown).

Possible deletion of a base during cloning of a gene copy

The amino acid sequence data of Ward and Dopheide (14) enabled us to determine the correct reading frame for the nucleic acid sequence of the ds DNA copy of 29C HA. However, reading backwards in this frame towards the N-terminus of HA1, our initial sequence for 29C contained an in-phase termination codon at bases 95-97 (Fig. 3a) and no in-phase ATG codon. The sequence of both strands of the cloned insert agreed in this respect (data not shown). We therefore attempted to confirm the sequence of this region directly by using a MboII/Hae III fragment (bases 45-76 of the cloned insert) as a primer for cDNA synthesis, with 29C genome RNA as a template (17). The sequence of the HA gene thus derived included an extra A residue at position 107 in the plus strand (Fig. 3b) which provided a continuous reading frame back to the ATG codon at bases 30-32 and yielded an amino acid sequence compatible with that determined for the N-terminus of mature HA from A/Mem/102/72 (26). We also determined the nucleotide sequence in this region for C55, another plasmid containing a dsDNA copy of the HA gene from 29C, isolated with C89 from the same E.coli RRI transformation. Unlike C89, this gene insert contained the A/T base pair at position 107 (data not shown).

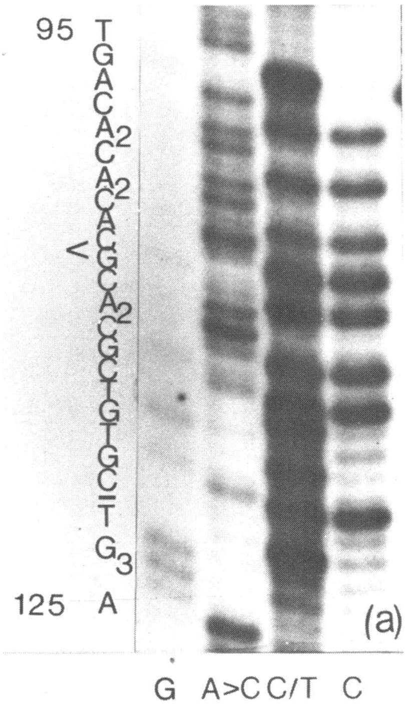
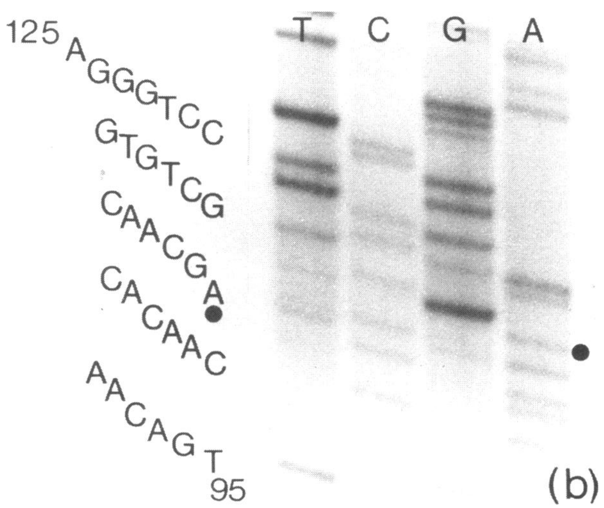


Figure 3. Comparison of (+) strand DNA sequences between bases 95-125. (a) A Hinf I/Hae III fragment labelled at the Hinf I site was sequenced by the Maxam and Gilbert procedure (15). The position of the missing base is indicated (<). The base marked (-) at position 120 is a C residue and is part of an Eco RII restriction endonuclease site which is methylated when the hybrid plasmid is grown in E. coli RRI. (b) 29C genome RNA was used as a template for cDNA synthesis by reverse transcriptase using a MboII/Hae III primer. Sequence data was obtained by the "dideoxy" method (16). The apparent missing residue in the cloned DNA copy of the HA gene (see (a)) is indicated (●).



DISCUSSION

Apparent deletion of a base from the HA gene copy in plasmid C89. A comparison of the nucleotide sequences determined for HA genes (bases 95-125) from the (+) strand of the cloned gene copies in C89 and C55 with the sequence obtained directly from the genome RNA indicates that at position 107, a residue present in the gene is missing in the C89 gene copy. This region of the HA gene can be drawn in a hairpin configuration (Fig. 4) with a stability of -4 Kcal (27). The presence of multiple bands on the sequencing gel (Fig. 3 b) between positions 103 and 111 may indicate that the hairpin structure is sufficiently stable to present reverse transcriptase with some difficulty in negotiating the 3' proximal side of the base-paired region. We speculate, therefore, that the presence of this hairpin may result in incorrect copying of the RNA by reverse transcriptase. Both Porter et al. (1),in cloning the FPV HA gene and Richards et al.(28), in studying copies of chicken β-globin mRNA found evidence for altered and missing bases in cloned DNA. However, they attributed this to repair or incorrect copying of mismatched regions associated with the terminal loop priming second strand DNA synthesis.

While it is possible that the HA gene copy in C89 represents a variant gene present in the viral population, such a deletion mutant should be extremely rare, since the deletion would result in the premature termination of synthesis of the HA protein, and this would be lethal in the next generation. Because the reverse transcriptase lacks a 3' exonuclease which could edit mistakes, it is possible that errors may occur with low frequency during

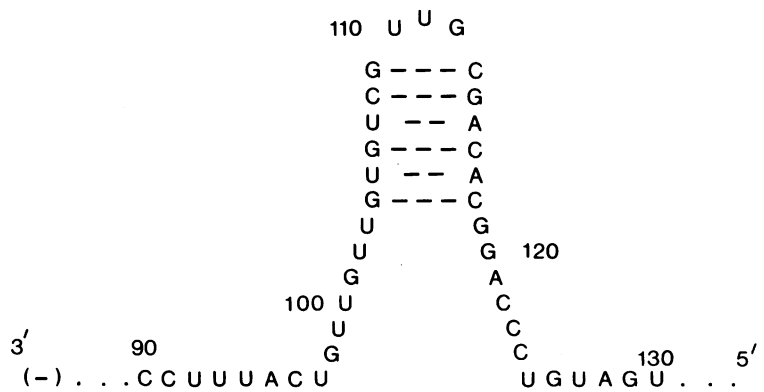


Figure 4. Structure of a hairpin loop which could form in the region of bases 100-120 of the gene.

the multi-step cloning procedure. Therefore, to guard against such errors when studying genes for which no protein sequence data are available, it may be necessary to derive nucleotide sequences from more than one cloned gene copy.

Structure of the HA gene from influenza of the Hong Kong subtype. Analyses by restriction enzyme mapping (7), nucleotide sequencing of the cloned HA gene copy and determination of the terminal sequence of the gene itself, revealed a length of 1765 nucleotides for the HA gene from the Hong Kong influenza strain 29C. This agrees with our previous estimate (1760 nucleotides) based on electrophoretic mobility (11) and compares with a length of 1742 nucleotides for the HA gene from the avian influenza strain FPV (Rostock) (1).

The arrangement of the HA genes from 29C and FPV are compared in Fig. 5. At the 3' end of the negative (genome) strand is a non coding sequence which appears to be completely transcribed into cRNA *in vitro* (23) and *in vivo* probably forms the 5' non-translated region of the mRNA. This section of mRNA may be subsequently modified *in vivo* if host-derived sequences and m^7G caps are attached (29).

Of the potential initiation codons in the (+) strand, only the one following the first 29 bases is in the correct phase to provide a continuous reading frame, which is also the frame prescribed by the known amino acid sequences for HA from the Hong Kong strain A/Mem/102/72 (14,26). The next AUG in this phase occurs 578 bases into the gene. Commencement of protein synthesis at bases 30-32 would produce a very hydrophobic peptide of 16 amino acids preced-

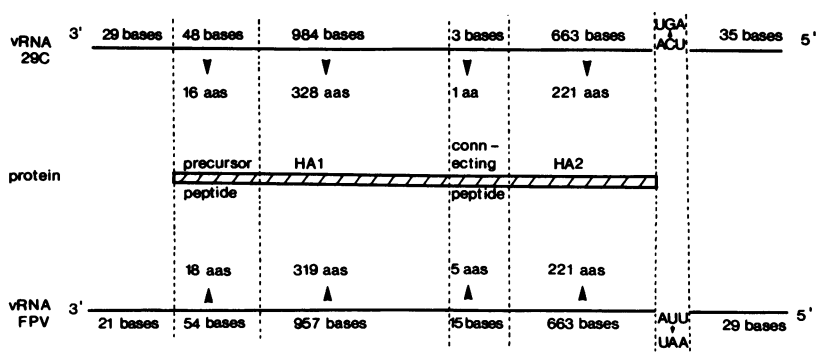


Figure 5. Comparison of the HA gene structures for Hong Kong and Fowl Plague viruses.

ing the glutamine residue (bases 79-81) found to be the N-terminal amino acid of the mature HA protein from A/Mem/102/72 (25).

The major and minor subunits (HA1 and HA2 respectively) of the mature HA protein appear to be generated by proteolytic cleavage of the primary translation product, with the loss of some amino acids connecting the two sections (30). Aligning the amino acid sequence found at the end of the HA1 and the beginning of HA 2 for influenza A/Mem/102/72 (15) with the amino acid sequence predicted by the HA gene from 29C, suggests that the connecting peptide consists of a single arginine residue. The HA subunits of A/Vic/3/75 are also linked by one arginine residue (31). In this respect, the HA of these strains resembles the H2-type HA from the Asian influenza strain A/Jap/305/57 (32) but differs from the FPV protein, where the HA subunits are connected in the immature protein by a basic pentapeptide (1).

The first in-phase termination codon (Fig. 5) is followed by only a short non coding sequence. How much of this sequence is transcribed into mRNA is not known, but it has been suggested that the U-rich sequence in the gene in this region may signal the end of transcription (1), providing a site for addition of poly A to the mRNA. Thus the 3' non-translated region of the mRNA following the termination codon could be as short as 14 bases in Hong Kong HA and 6 bases in FPV HA.

The amino acid sequence predicted from nucleotide sequence data for the HA gene of influenza A/Vic/3/75 (31) contained an additional asparagine residue following HA1 residue No. 8 (Fig. 2a). However, this additional residue may be unique to the particular isolate studied, since it is absent from H3-type HA1's in a total of six influenza strains isolated between 1968 and 1977. (Both and Sleigh, unpublished results).

Comparison of nucleic acid sequences of Hong Kong and FPV HA genes. The genes from the two subtypes have similar base compositions: for 29C A24%, G 20.5%, C23.5%, U 32% and for FPV, A24%, G 18.4%, C 23.8%, U 33.8%. Codon utilisation in the Hong Kong HA gene is similar to that for FPV, with some exceptions which may reflect the availability of isoacceptor tRNAs in the host, e.g. CUG is preferred for leu in the Hong Kong gene while FPV uses AAA for lys in preference to AAG (Table 1). The incidence of CpG dinucleotides is low for both genes, as noted previously for FPV (1).

Comparison of amino acid sequences predicted by the two genes. The amino acid sequence predicted from the nucleotide sequence for the 29C HA gene (Fig. 2) is largely identical to that found for the HA protein from A/Mem/102/72 (14,26). As for HA molecules from other influenza strains, HA1 has a high

Table 1: Codon utilization in HA genes from Hong Kong and Fowl Plague Influenza Viruses. FPV data is in brackets below the corresponding figure for 29C.

		U	C	A	G			U	C	A	G
		9	6	9	6			9	6	8	0
	U	(14)	(4)	(7)	(7)		U	(9)	(3)	(7)	(1)
		13	4	9	12			3	4	3	2
	C	(12)	(4)	(8)	(9)		C	(4)	(4)	(4)	(0)
U						C					
		1	10	0	1			7	7	16	1
	A	(6)	(13)	(1)	(0)		A	(4)	(6)	(14)	(4)
		7	2	0	12			16	3	8	3
	G	(9)	(1)	(0)	(8)		G	(8)	(3)	(11)	(3)
		14	16	22	3			11	10	13	10
	U	(15)	(13)	(23)	(11)		U	(9)	(12)	(17)	(5)
		18	6	21	14			4	5	20	7
	C	(9)	(12)	(14)	(7)		C	(6)	(3)	(9)	(10)
A						G					
		14	13	19	10			9	11	16	13
	A	(16)	(15)	(24)	(14)		A	(5)	(16)	(28)	(20)
		9	4	11	11			6	2	13	14
	G	(11)	(1)	(7)	(8)		G	(10)	(1)	(10)	(15)

proline content relative to HA2. Also remarkable is the similarity with other strains in the number and distribution of cysteine residues in the 29C protein (9 in HA1, 8 in HA2) (1,14,30). Only one near the end of HA2 has no counterpart in the FPV molecule. If the FPV and Hong Kong HA amino acid sequences are aligned for maximum homology using the cysteine residues, seven of the ten proline residues in the C-terminal half of the HA1 are also conserved between the subtypes. This suggests that the shape of this part of the molecule is not permitted to vary extensively.

Potential sites for carbohydrate attachment (Fig.2), occurring (by analogy with HA from the Asian subtype) at sequences of the type Asn-X-Thr (30),are not conserved between subtypes. With the cysteine residues aligned, the sites at positions 22 and 38 in 29C are equivalent to those at 12 and 28 in FPV (1).

With the cysteine residues aligned, there is approximately 38% amino acid conservation in HA1 between FPV and 29C. In HA2 there is 65% homology, but in more than half of the 145 cases where the amino acid is conserved a different codon is used; 69 differ by one base, 5 differ by two

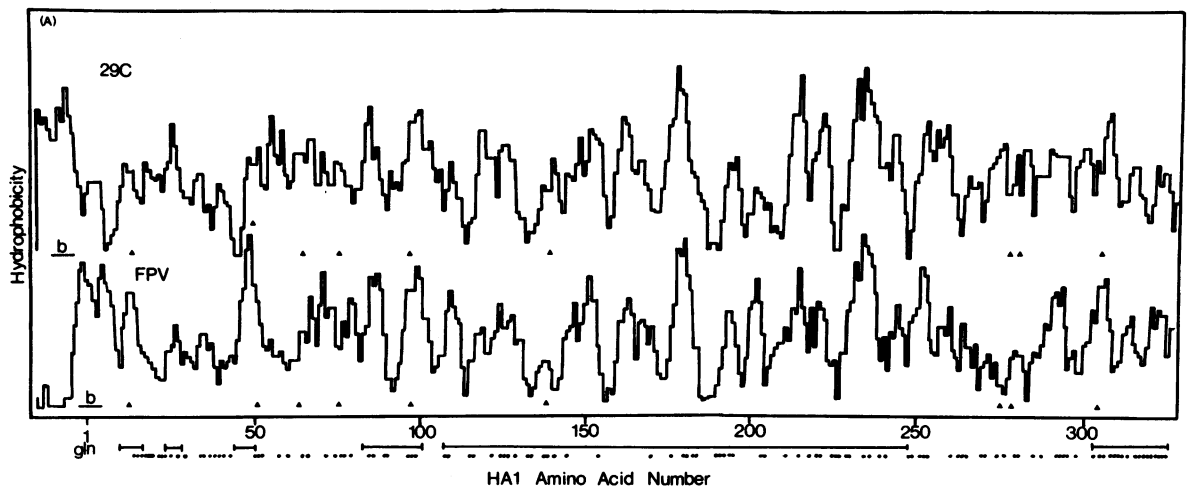


FIGURE 6. Relative hydrophobicities of the HA amino acid chains from FPV and strain 29C (a) HA1, (b) HA2. Computer-generated plots show hydrophobicities (20,21) as a moving average over five amino acids. In HA1 the two sequences were aligned to give maximum amino acid homology by introducing a single gap after residues 168 and 276 in 29C, and after residue 252 in FPV. Regions of similar hydrophobicity profile are indicated by solid lines below the figures, with homologous amino acids indicated by a dot. Cys residues are shown (▲) and a base line for each curve is indicated b.

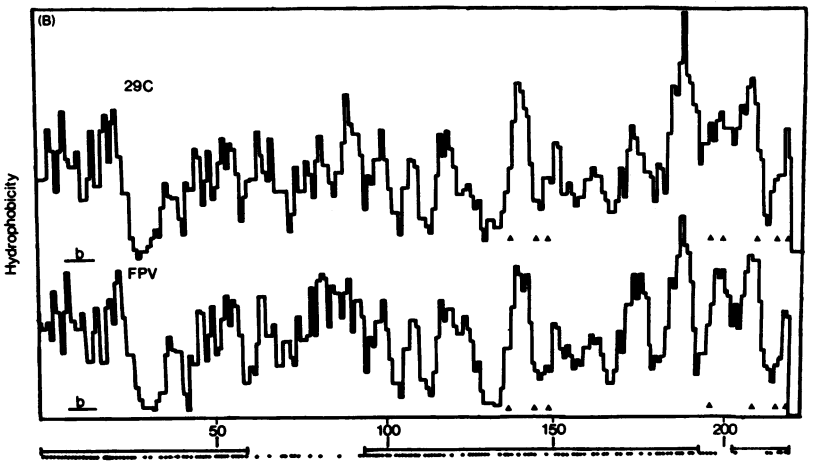


Fig. 6b

and in one case a serine uses an AGC instead of a UCA codon. Some areas of HA2 show a particularly high degree of amino acid conservation, e.g. the N-terminal region. In addition, in some areas of HA2 where the amino acid sequence is different, the character of the protein tends to be preserved. Figure 6 shows an analysis of the degree of hydrophobicity of different areas of the HA protein from 29C and FPV. In the C-terminal region of HA2, thought to be involved in anchoring the HA to the viral lipoprotein membrane (30), both proteins are highly hydrophobic in character, even though between residues 199 and 212, only one out of 13 amino acids is conserved. This effect extends to other regions of the HA as well. For example, the precursor peptides, cleaved from HA during maturation, differ in length and sequence among FPV, 29C and viruses from the H2 subtype (1, 32, 33); but are all hydrophobic in character. Also notable is the area between HA1 residues 85 and 240 of 29C for which the hydrophobicity profile is broadly similar to the equivalent area in HA1 of FPV, although the amino acid sequences show only 32% homology. This type of analysis suggests that amino acid divergence between HAs from different subtypes may be strictly limited in some areas to those changes which do not significantly disturb the local environment, while in other areas (e.g. residues 1-100 of HA1) little constraint is apparent. As sequence information on HA molecules from further influenza subtypes becomes available, it should be possible to identify regions of the protein which are essential to maintain HA structure and function. In addition, comparison in this way of closely related proteins from viruses

of the same subtype may help to identify the amino acid changes which are important in altering viral antigenicity.

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